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Serine isotopomer analysis by ^{13}C-NMR defines glycine-serine interconversion in situ in the renal proximal tubule

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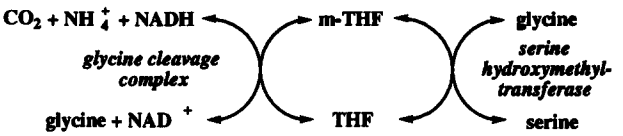
Abstract

[2-^{13}C]glycine metabolism was studied in freshly isolated rat renal proximal tubules. Mitochondrial coupling of the glycine cleavage complex (GC) and serine hydroxymethyltransferase (SHMT) was confirmed by the formation of three serine isotopomers, [2-^{13}C]-, [3-^{13}C]- and [2,3-^{13}C]serine, detected by ^{13}C-NMR. Incubation with different fractions of ^{13}C-labelled glycine altered the labelling pattern of the serine isotopomers predictably and allowed calculation of the ^{13}C-labelled fractions of total glycine and methylene in N^5,N^{10}-methylenetetrahydrofolate (m-THF) available for serine metabolism. Within 20 min there was a fall in labelled glycine (to 42 ± 3, 68 ± 3 and $93 \pm 2\%$, ($n = 4$, mean $\pm$ S.D.) from 50%, 75% and 100% ^{13}C-labelled added glycine respectively), followed by a slow rate of endogenous glycine formation for up to 80 min incubation. The C2 of glycine was the source of more than 90% of the methylene group of m-THF formed. Gas chromatography-mass spectroscopy (GC-MS) showed that greater than 50% of serine formed was unlabelled. GC and SHMT proceeded in the direction of serine formation. Serine isotopomer analysis by NMR and GC-MS allowed the actions of GC and SHMT and de novo contributions to glycine, serine and m-THF to be monitored in situ in fresh renal proximal tubules.

Keywords: Glycine; Serine; Nuclear magnetic resonance; Kidney; Proximal tubule

1. Introduction

Recent observations that glycine, and to a lesser extent alanine, protect isolated renal proximal tubules, cultured renal cells and possibly the intact kidney against hypoxic and toxic injury [1–7] have stimulated interest in glycine metabolism. The two principle enzymes involved in glycine and serine interconversion are serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) and glycine cleavage complex (GC, EC 2.1.2.10). Studies of these enzymes in liver suggest that SHMT catalyses the breakdown of serine and that glycine catabolism occurs via GC [8]. These reactions maintain the one-carbon pool, essential in many cellular synthetic pathways. The combination of these reactions in the reverse direction allows the synthesis of serine from two molecules of glycine:



Scheme 1

Serine is released into the circulation by the kidney, indicating net renal serine formation. Renal arteriovenous differences have demonstrated that glycine is quantitatively important as a serine precursor [9], suggesting serine formation by GC and SHMT in the kidney. Detailed investigation of glycine-serine interconversion has been made in freshly isolated renal proximal tubules. While SHMT is present in both mitochondria and cytosol, GC activity is restricted to the mitochondria [10,11]. The inner mitochondrial membrane is impermeable to reduced folate coenzymes [12,13], therefore serine formation via SHMT utilising m-THF formed from glycine must be exclusive to mitochondria. Investigations of purified liver SHMT have shown differences between cytosolic and mitochondrial

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SHMT, including greater affinity for glycine in the mitochondrial enzyme [14] and dependence of kinetic parameters on the number of glutamates bound to m-THF [15]. These studies suggest similar rates for the forward and reverse reactions of SHMT. Investigations of [2-^{14}C]glycine metabolism in rat kidney cortical slices demonstrated that the ratio of glycine utilisation:serine formation:ammonium formation:$^{14}CO_2$ liberation is 2:1:1:1 [16]. Therefore, m-THF formed from glycine is utilised by SHMT and is not oxidised further [10,14,15], suggesting tight coupling of GC and SHMT reactions.

The presence of both GC and SHMT enzymes in a tissue should lead to the formation of [2-^{13}C]serine, [3-^{13}C]serine and [2,3-^{13}C]serine from [2-^{13}C]glycine which can be detected as unique peaks by ^{13}C-NMR. This is not easily achieved using GC-MS, which cannot differentiate between [2-^{13}C]- and [3-^{13}C]serine, or by ^{14}C-studies which would require isolation of each carbon within serine. These three serine isotopomers were recently detected in *Saccharomyces cerevisiae* by ^{13}C-NMR [17]. However, the single ^{13}C-NMR study of [2-^{13}C]glycine metabolism by renal cells, that of cultured LLC-PK_1/Cl_4 cells [18], reported only [2-^{13}C]serine formation, which suggests that the GC was absent. In this study we examined serine formation from [2-^{13}C]glycine in freshly isolated rat renal cortical tubules and present a novel method of determining the fraction of ^{13}C-labelled glycine and m-THF available for serine metabolism for comparison with the fraction of ^{13}C-labelled glycine in the total sample as measured by GC-MS. The fraction of methyl groups in m-THF derived from glycine could also be calculated. Comparison of serine isotopomer analysis and GC-MS should allow the relative rates of the forward and reverse direction of SHMT and GC and de novo formation of glycine and serine to be estimated in intact proximal tubules.

2. Experimental

2.1. Proximal tubule isolation

Male Wistar rats, weighing 400–550 g, with free access to water and fed ad libitum with rat and mouse cubes (NORCO, Lismore, NSW, Australia), were used in all experiments. The rats were anaesthetised with pentobarbitone sodium (60 mg/kg i.p.). The abdominal aorta was exposed to allow antegrade perfusion of the kidneys with 50 ml of solution A (calcium-free Hanks' balanced salt solution buffered with Hepes and containing 5 mM glucose, 4 mM lactate and 1 mM alanine). The kidneys were decapsulated, removed and placed in solution A on ice. Cortical tubules were then isolated by a modification of the method of Vinay et al. [19]. While keeping the kidney cold, the cortex was dissected off, weighed, finely minced, then digested for 50 min in 1.5 mg/ml collagenase (Sigma type II) in solution B (solution A supplemented with 0.1 mM $CaCl_2$ and 2.5% BSA, Pentex, fraction V, Miles Laboratory, California, USA) at 37°C under 95%O_2/5%CO_2 in a hooded shaking water bath. The digested tissue was filtered, washed twice with cold solution B then resuspended in 35% Percoll (Pharmacia, Uppsala, Sweden) in Krebs Henseleit saline (KHS) containing 5 mM glucose, 4 mM lactate and 1 mM alanine and centrifuged at $30000 \times g$ for 30 min at 4°C. Proximal tubule segments were harvested from the thick bands near the bottom of the tube. A sample of freshly isolated tubules was kept for assay of hexokinase [20], phosphoenolpyruvate carboxykinase [21] and for microscopic examination. Prior to incubation with ^{13}C-substrates, tubules were washed sequentially at 4°C with solution B, with KHS and with the incubation medium (one wash each).

2.2. Experimental protocol

Incubation was commenced by adding tubules, 1.5 to 2 ml loosely packed tubules (cytocrit ~ 30%, 225–270 mg protein) to the incubation medium in a screw top conical flask, top gassed with 95%O_2/5%CO_2 (O_2/CO_2) at 2 l/min for 4 min in a shaking water bath at 37°C and sealed. The flask was regassed following addition or removal of samples. For sequential studies, purified proximal tubules were added to 40 ml KHS containing 5 mM glycine containing 75% ^{13}C-labelled [2-^{13}C]glycine (C/D/N Isotopes, Quebec, Canada). A 5-ml sample was taken immediately (0 min) and at 5, 10, 20, 40 and 80 min. The samples were snap-frozen in liquid N_2 and stored at −70°C. For studies comparing the effect of incubation with variable fractions of ^{13}C-labelled glycine, tubules were added to 15 ml KHS containing 5 or 10 mM glycine, either as 100%, 75%, or 50% ^{13}C-labelled [2-^{13}C]glycine with [2-^{12}C]glycine making up the balance. The tubules were then incubated for 20 min at 37°C. Following incubation, the sample was centrifuged, and the supernatant and pellet were snap-frozen in liquid N_2 and stored at −70°C. Samples were extracted with perchloric acid (PCA) for ^{13}C-NMR analysis as described below.

^{14}C-Inulin (0.15 μCi, Amersham International, Amersham, UK) was added 10 min before the end of all incubations for estimation of the fraction of extracellular water, from which sample wet weight could then be calculated. Samples were taken at the completion of incubation for assay of protein, ^{14}C-inulin and LDH.

2.3. Tubule integrity

Tubule cell integrity was assessed by measuring the release of lactate dehydrogenase (LDH) into the incubation medium. LDH was assayed after Bergmeyer et al. [22] and expressed as the percent of activity in the supernatant relative to the total sample following addition of Triton X-100. The fraction of viable cells was assumed to equal (100 − %LDH released)/100.

2.4. Protein determination

Protein was measured by a modification [23] of the method of Lowry [24]. BSA was used as the protein standard.

2.5. Sample extraction

Samples were weighed and placed on ice. Two volumes of 12% PCA were added to the samples, which were then homogenised by three 3-s bursts of an IKA homogeniser (type 25, Janke and Kunkel, Staufen, Germany). The samples were centrifuged at 4°C and the supernatant stored on ice. The pellet remaining from each sample was extracted a second time with PCA. Following centrifugation, both supernatant samples for each experiment were pooled, neutralised using 3 M KOH, centrifuged, then freeze-dried and stored at −20°C. Samples were kept at 4°C throughout the extraction.

2.6. NMR sample preparation

One ml D_2O (99.9 atom% D, Sigma Chemicals, St. Louis, USA) and 4 ml H_2O were added to each lyophilised sample and the pH adjusted to 7.0 with KOH. The sample was then stored at 4°C until analysis. Immediately prior to the NMR. measurement, 3 ml of the supernatant was transferred to a 10 mm NMR tube and 150 μl of 1.8 M tetramethylsilylpropanoic acid (TSP, MSD Isotopes, Montreal, Canada) was added to a final concentration of 85.7 mM. Ethylenediaminetetra-acetic acid (EDTA, 1 mg) was added to each sample. ^{13}C-NMR spectra were acquired using a Bruker AMX500 spectrometer. Standard data acquisition parameters for partially relaxed spectra used were: frequency 125.77 MHz; temperature 303°K, repetition interval 2 s (acquisition time 0.49 s; recycle delay 1.5 s, 7 μs pulse length). The WALTZ sequence was used for proton decoupling [25].

2.7. Isotopomer analysis

When the incubation medium contains both [2-^{13}C]glycine and unlabelled glycine (i.e., [2-^{12}C]glycine), four isotopomers of serine should be formed by the following reactions:

$$[2-{}^{13}C]glycine + [{}^{13}C]m\text{-}THF \rightleftharpoons [2,3-{}^{13}C]serine \quad (1)$$

$$[2-{}^{13}C]glycine + m\text{-}THF \rightleftharpoons [2-{}^{13}C]serine \quad (2)$$

$$glycine + [{}^{13}C]m\text{-}THF \rightleftharpoons [3-{}^{13}C]serine \quad (3)$$

$$glycine + m\text{-}THF \rightleftharpoons serine. \quad (4)$$

The ratio of the reaction products of Eqs. (1) and (3) are independent of the fraction of labelled m-THF and may be written as:

$$[2,3-{}^{13}C]serine/[3-{}^{13}C]serine = [2-{}^{13}C]glycine/glycine, \quad (5)$$

so that the fraction of glycine which is [2-^{13}C]-labelled (F_{gly}) is given by [26]:

$$F_{gly} = [2,3-{}^{13}C]serine/([2,3-{}^{13}C]serine + [3-{}^{13}C]serine). \quad (6)$$

Reactions 1 and 2 are similarly dependent on the ^{13}C-labelled m-THF ratio and independent of the labelled glycine ratio. Therefore the fraction of labelled m-THF (F_m) can be obtained from:

$$[2,3-{}^{13}C]serine/[2-{}^{13}C]serine = {}^{13}C-m\text{-}THF/m\text{-}THF, \quad (7)$$

hence

$$F_m = [2,3-{}^{13}C]serine/([2,3-{}^{13}C]serine + [2-{}^{13}C]serine). \quad (8)$$

If m-THF is derived exclusively from glycine, the percentage of labelled m-THF will equal the fraction of the labelled glycine. We can determine the percentage of m-THF formed from glycine ($F_{m(GC)}$) by:

$$F_{m(GC)} = F_m/F_{gly} \quad (9)$$

2.8. NMR quantitation

Peak assignment was based on published data, spectra of aqueous standards acquired under similar conditions and by superposition of peaks in experimental samples of proximal tubule extracts. The FID was Fourier-transformed with 2 Hz line broadening. The Bruker deconvolution package with 100% Lorentzian line shape simulation was used to determine peak integrals. The only case in which the deconvolution package had difficulty simulating peaks was for the serine singlets in the 100% labelled glycine experiments. Unlabelled glycine is required for singly ^{13}C-labelled serine formation. Since no exogenous unlabelled glycine was added, only very small amounts of singly ^{13}C-labelled serine were formed. The line width, and hence integral, assigned to these singlet peaks was much greater than the standard ^{13}C peak line width, as a consequence of low signal-to-noise (S/N) and the overlapping shoulders of the doublet peaks (Fig. 3). Independent investigation of this effect suggested that peaks with S/N levels below 10, start to demonstrate this effect (data not shown). Since both the singlet and doublet are formed from the same carbon, similar line widths are expected.

Peak intensities were standardised to allow for this variation by dividing the integral of each peak by its own line width. When peak quantitation was required, uncorrected peak integrals were used, except the singlet peaks in the 100% labelled glycine experiments were divided by their line width and then multiplied by the average line width of the carbon doublet peaks.

For determination of F_{gly}, [2,3-^{13}C]serine was determined from the sum of the intensities of the C3-serine doublet peaks and [3-^{13}C]serine from the C3-serine singlet peak. For determination of F_m, [2,3-^{13}C]serine was estimated from the sum of the C2-serine doublet peaks and [2-^{13}C]serine as the C2-serine singlet peak. Since only singlets and doublets from the same carbon were compared, correction for T_1 and nOe differences between carbons was not required. Bonded ^{13}C atoms introduce an additional dipole relaxation mechanism with the potential to influence the T_1 of the carbon atoms and hence the nuclear Overhauser enhancement potentially introducing quantitation errors. However, previous investigations of this effect have demonstrated that the ^{13}C-^{13}C dipole contribution to the nuclear Overhauser enhancement for carbons with bonded protons (as in the 2- and 3-position of serine) is less than 1% [27]. However, when carbon atoms are not protonated, e.g., carboxylic acid groups, the contribution may be 25–30% [27]. Hence, previous metabolic studies utilising isotopomer analysis only derive results from protonated carbons, e.g., the 2, 3 and 4-positions of glutamate but not position 1 or 5 [28]. Similarly, the relative intensities of multiplets derived from protonated carbons in the 2- and 3-position of serine in our experiments are not significantly influenced by ^{13}C-^{13}C dipole interaction.

Glycine and serine concentrations were estimated from their peak intensities relative to TSP which was in turn calibrated against standard curves using PCA-extracted samples with known glycine and serine concentrations measured under identical conditions. Separate calibration curves were used for [2-^{13}C], [3-^{13}C]serine and [2-^{13}C]glycine. For [2,3-^{13}C]serine the 2- and 3-position doublets were calibrated against the 2- and 3-position singlet curves. Since one molecule of [2,3-^{13}C]serine contains two atoms of ^{13}C, the average of the doublet concentrations represents the amount of serine which is doubly ^{13}C-labelled. The sum of the doublet concentrations represents the total amount of ^{13}C incorporated into [2,3-^{13}C]serine. Summarising, the amount of ^{13}C-labelled serine formed was taken as the sum of the concentrations of [2-^{13}C]serine, [3-^{13}C]serine plus the average of the 2-carbon and 3-carbon doublets. Total incorporation of ^{13}C-label into serine (for comparison with the decrease in [2-^{13}C]glycine) was determined from the sum of the concentrations from [2-^{13}C]serine, [3-^{13}C]serine plus both serine doublets. During a 20-min incubation serine formation was expressed as μmol · g wet wt^{-1} and μmol · g protein^{-1}. Rates from sequential studies were determined as μmol · g protein^{-1} per h for two time periods, 0–5 min and 20–80 min. When monitoring time-dependent changes, peak intensities were corrected for cell viability by dividing the change in intensity between time points by the average cell viability at these points and then adding the quotient to the peak intensity of the first of the two time points.

2.9. GC-MS analysis of ^{13}C enrichment of serine

Serine and glycine ^{13}C enrichment was performed using a Hewlett-Packard 5840 gas chromatography unit using a DB-1 bonded phase capillary column and a Hewlett-Packard 5985B mass spectrometry system operating in the selected-ion-monitoring data acquisition mode. Samples were prepared as follows, a 1:1 slurry of cation exchange resin (Dowex AG50 × 8; 200–400 mesh, Bio-Rad Laboratories, Hercules, CA) and deionised water was added to a disposable column to provide a 2-cm resin bed. The resin was washed twice with 5 ml of deionised water and kept moist. The NMR sample (0.5 ml) was acidified with 0.1 M HCl to pH 2 and added to the resin bed. The column was washed with 2 × 5 ml of deionised water and the eluant discarded. The amino acids were eluted with 2 × 1 ml of 3 M NH_4OH. The eluant was collected and dried. Tertiarybutyldimethylsilyl-derivatives of the amino acids were prepared by adding *N*-methyl-*N*-(tertbutyldimethylsilyl)-trifluoroacetamide:acetonitrile (1:1, both compounds from Pierce, Rockford, IL) to the dried residue and heating to 100°C for 2 h. Ions of mass 246.1 and 247.1 were monitored for glycine and ions of mass 390.2, 391.2 and 392.2 were monitored for serine.

2.10. Statistical methods

Results are expressed as mean ± S.D., statistical differences between points within an experiment were determined using a paired Student *t*-test, while differences between different experiments were determined using an unpaired Student *t*-test.

3. Results

3.1. Proximal tubule purity and integrity

By microscopy, 95% of purified cortical tubules were short lengths of proximal tubules, while the remainder were principally segments of the thick ascending limb of the loop of Henle. The specific activity of phosphoenolpyruvate carboxykinase was 37.3 ± 8.0 μmol · min^{-1} per g protein (mean ± S.D., $n = 10$), and the activity of hexokinase, 27.8 ± 4.6 μmol · min^{-1} per g protein ($n = 10$), confirming that the tubules were primarily of proximal origin. The average LDH released into the medium with time for tubules incubated in 5 mM glycine is shown in Table 1. LDH release into the medium at 20 min for

Table 1
Tubule viability and F_{gly} following incubation with 5 mM 75% [2-^{13}C]glycine

Incubation time (min)	0	5	10	20	40	80
% LDH release	4.7	5.3	5.3	6.8	12	30
F_{gly}(%)		68.8	67.9	67.8	66.8	65.8

Dependence of tissue viability, expressed as percent of LDH released into the medium, on incubation time.
The fraction of ^{13}C-labelled glycine was also calculated for each time point ($n = 2$).

tubules incubated with different fractions of 5 mM labelled glycine was $7.7 \pm 1.5\%$ ($n = 12$), which is similar to values reported for incubation of proximal tubules with other substrates in addition to glycine [1].

3.2. Incubation of proximal tubules with [2-^{13}C]glycine

A typical ^{13}C-NMR spectrum from proximal tubules incubated in 5 mM [2-^{13}C]glycine for 20 min is shown in Fig. 1. The spectrum shows the incorporation of the ^{13}C-label from [2-^{13}C]glycine (42.8 ppm) into the three isotopomers of serine: the [2-^{13}C]serine singlet at 57.7 ppm; the [3-^{13}C]serine singlet at 61.5 ppm and the [2,3-^{13}C]serine with doublet peaks at 57.6 and 57.8 ppm for C2-serine and at 61.4 and 61.6 ppm for C3-serine. The intense peak at −1.7 ppm and multiplets at 13 and 32 ppm are from the TSP reference. Small peaks at 45.9 ppm and 62.1 ppm were often observed but remain unassigned.

The sequential incorporation of ^{13}C-label from [2-^{13}C]glycine into serine is shown in Fig. 2. Peak intensities were averaged from two experiments, each corrected for sample weight, protein content and the percentage of viable cells estimated from the LDH release into the medium. The fraction of ^{13}C-labelled glycine (F_{gly}) determined by

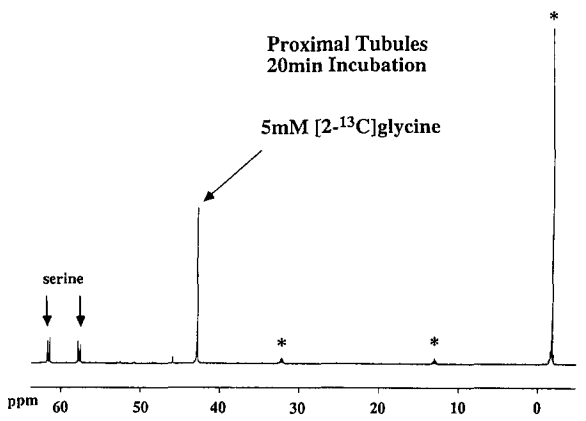

Fig. 1. ^{13}C-NMR spectrum of glycine metabolism by renal proximal tubules. Purified proximal tubules were incubated with 5 mM [2-^{13}C]glycine for 20 min. Samples were extracted with perchloric acid, freeze-dried then reconstituted with 20% D_2O in H_2O for ^{13}C NMR acquisition. (*)sodium 3-(trimethylsilyl) propionate-2,2,3,3-D_4 reference.

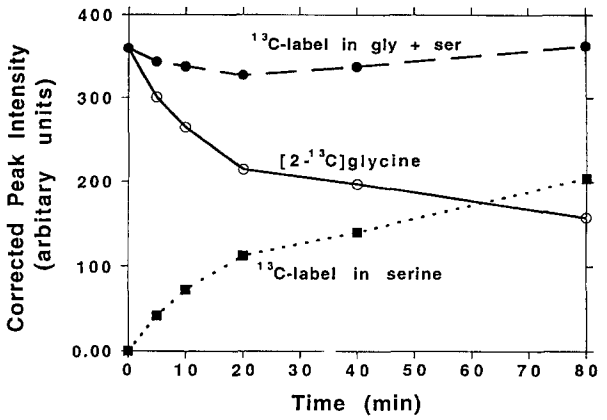

Fig. 2. Time-dependent incorporation of ^{13}C-label from glycine into serine. [2-^{13}C]glycine (○), ^{13}C-label incorporated into serine (■) and the sum of ^{13}C label in glycine and serine (●) were determined during incubation of proximal tubules with 5 mM 75% ^{13}C-labelled glycine ($n = 2$). The peak intensities of glycine and serine at each time point were corrected for sample weight and protein content. The formation rates of ^{13}C-labelled serine from 0–5 min and 20–80 min are 340 μmol·g protein^{-1} per h and 61 μmol·g protein^{-1} per h respectively.

application of Eq. 6 to the serine isotopomers is shown in Table 1. The decrease in the mean values of F_{gly} for the two time series experiments was not significant over the 80-min incubation period. No major metabolites of glycine other than serine were observed in the ^{13}C-NMR spectrum (Fig. 1). This is supported by the constant sum of ^{13}C-label in glycine and serine (Fig. 2).

3.3. Varying the fraction of ^{13}C-labelled glycine

An incubation time of 20 min was chosen to ensure good cellular integrity and stable reaction conditions. Typical spectra from tubules incubated in 10 mM glycine showing the relationship between the ratio of doubly and singly labelled serine and the fraction of initial labelled glycine added are shown in Fig. 3. Incubating proximal tubules with 100% [2-^{13}C]glycine formed mainly [2,3-^{13}C]serine. As unlabelled glycine was added, reducing the fraction of ^{13}C-labelled glycine to 75% or 50%, the singlets formed by [2-^{13}C]serine and [3-^{13}C]serine predominated over [2,3-^{13}C]serine. The mean fraction (±S.D.) of ^{13}C-labelled glycine (F_{gly}) calculated from the serine isotopomers and that determined directly by GC-MS for tubule samples incubated with 5 mM glycine in 4 separate experiments are shown in Table 2. There was no significant difference between the NMR and GC-MS results for each group. The reduction by 7–8% in the fraction of ^{13}C-labelled glycine following 20 min incubation suggests formation of unlabelled glycine or dilution with pre-existing unlabelled glycine. Estimates of the fraction of labelled m-THF are similar to although lower than the fraction of labelled glycine at 100% and 75% initial fraction of ^{13}C-

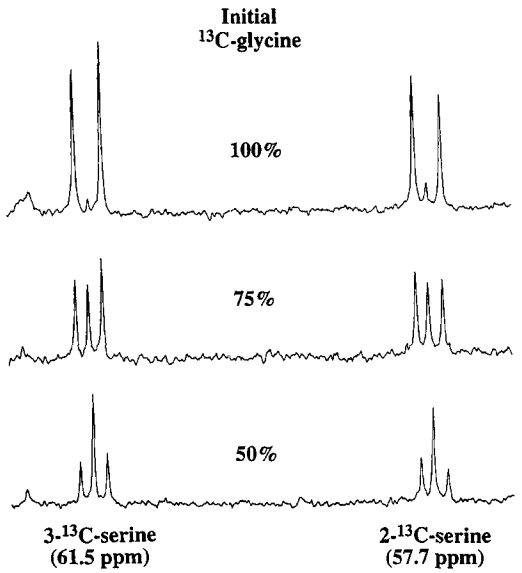

Fig. 3. Dependence serine ^{13}C-NMR doublets and singlets on initial fraction of ^{13}C-labelled glycine. Freshly isolated proximal tubules were incubated for 20 min with 10 mM glycine, which was either 100%, 75% or 50% ^{13}C-labelled.

labelled glycine (Table 2). This is consistent with the high calculated fraction of m-THF derived from glycine via GC (Table 2), which indicates that the C2 of glycine is the major source of the methylene group in m-THF in this preparation. Ultimately this methylene becomes the C3 carbon of serine via addition to a second glycine in the reaction catalysed by SHMT.

The fraction of serine containing only ^{12}C carbon atoms, singly ^{13}C-labelled carbon atoms, and doubly ^{13}C-labelled carbons determined from GC-MS are presented in Table 3. The fraction of unlabelled serine was consistently slightly greater than 50%, even in experiments where the only substrate added was 100% ^{13}C-labelled glycine. When the fraction of unlabelled glycine was increased to 50%, the fraction of unlabelled serine increased. The proportion of singly and doubly labelled serine determined by GC-MS changed similarly to that determined by NMR with a decrease in doubly labelled serine and an increase in the singly labelled serine as the fraction of ^{13}C-labelled glycine was decreased. Thus approx. 50% of serine formed in proximal tubules incubated with glycine as a single substrate was not derived from this glycine but from some other source or was already present at the start of incubation.

Table 2
Comparison of isotopomer analysis and GC-MS data

	Initial ^{13}c-labelled glycine		
	100%	75%	50%
Glycine formation			
F_{gly}(^{13}C-NMR)	93±2	68±3 [a]	42±3 [a]
Glycine enrichment (GC-MS)	86±7	66±6 [a]	42±4 [a]
m-THF Formation			
F_m(%)	87±3 [b]	64±2 [ab]	42±0.7 [a]
F_m(GC)}(%)	94±3	95±2	99±7

Estimates of ^{13}C-labelling of glycine determined from serine isotopomer anaysis (^{13}C-NMR) and from direct measurement (GC-MS) following incubation of proximal tubules with ^{13}C-labelled and unlabelled glycine ($n = 4$, mean ± S.D.).
[a] Significant difference compared to previous column ($P < 0.05$).
[b] Significant difference compared to F_{gly} ($P < 0.05$).

Table 3
Serine production and ^{13}C-labelled distribution

	Initial ^{13}C-labelled glycine		
	100%	75%	50%
^{13}C-Serine formation			
μmol · g wet wt^{-1}	17±8	13±3	8.4±1.7
μmol · g protein^{-1}	70±21	49±10	37±6
Serine ^{13}C-enrichment (%)			
Unlabelled	53±3	53±3	58.7±1 [a]
Singly ^{13}C-labelled	13±3	20.5±5 [a]	23±0.5
Doubly ^{13}C-labelled	33.4±10.2	26.2±1.6 [a]	18±1 [a]

Following 20 min incubation, serine formation determined from ^{13}C-NMR, while serine ^{13}C-enrichment was determined by GC-MS ($n = 4$, mean ± S.D.).
[a] Significant difference compared to previous column ($P < 0.05$).

4. Discussion

The combined action of GC and SHMT in freshly isolated proximal tubules was confirmed by the formation of [2-^{13}C]serine, [3-^{13}C]serine and [2,3-^{13}C]serine from [2-^{13}C]glycine. Glycine metabolism effectively proceeded only to serine in this preparation of renal proximal tubules since other major peaks were not detected. This was supported by the time course of these reactions, in which the sum of ^{13}C-label in glycine and serine remained constant over 80 min (Fig. 2). Previous investigations of rat kidney cortical slices have found no evidence of serine utilisation [16]. After 20 min the rate of serine formation in our studies became constant (Fig. 2) at 61 μmol · g wet wt^{-1} per h (equivalent to 380 μmol · g dry wt^{-1} per h). This is much greater (allowing for differences in wet and dry weights) than previously reported for Percoll purified rat proximal tubules incubated with 5 mM glycine for 30 min (62.6 ± 11.4 μmol · g dry wt^{-1} per h) [29].

In contrast to our studies in freshly isolated rat proximal tubules, cultured porcine proximal tubular cells (LLC-PK_1/Cl_4) incubated with 7 mM [2-^{13}C]glycine show neither [3-^{13}C]serine nor [2,3-^{13}C]serine [18], suggesting that GC was absent or inactivated in this cultured cell line. An intense peak at 60.9 ppm in the PCA extract of LLC-PK_1/Cl_4 cells was assigned to [2-^{13}C]L-threonine [18] and led to the suggestion that SHMT catalyses the formation of threonine from glycine and acetaldehyde. Addition of threonine to our experimental samples formed a new peak

(61.7 ppm) not present in the initial spectra and partially overlapping the downfield 3-serine doublet. Reassigning the reported threonine peak at 60.9 ppm to [3-^{13}C]serine does not confirm GC activity in LLC-PK$_1$/Cl$_4$ cells, since [3-^{13}C]serine formation requires combined GC and SHMT activity, which would also form [2,3-^{13}C]serine and this was not present in the spectrum. The LLC-PK$_1$/Cl$_4$ cells were also reported to form ß-hydroxybutyrate at 45.9 ppm [18], which suggests that glycine and serine are precursors for the glycolytic pathway. Possible pathways for conversion of serine to acetyl-CoA are the non-phosphorylated pathway (via serine aminotransferase, EC. 2.6.1.51) and serine dehydratase (EC. 4.2.1.13) which are essentially restricted to the liver [30]. Although we usually obtained a small peak at 45.9 ppm, the addition of ß-hydroxybutyrate gave an independent peak at 47.3 ppm. Furthermore, we could not detect ß-hydroxybutyrate in samples analysed by high-performance liquid chromatography (data not shown). The small peak at 45.9 ppm remains unassigned. Our results from freshly isolated tubules suggest LLC-PK$_1$/Cl$_4$ cells have dedifferentiated with respect to glycine metabolism in normal renal proximal tubules. Other metabolic alterations, including increased glycolytic activity and decreased oxidative metabolism have been reported for primary renal proximal tubule cultures maintained in stationary culture [31].

These ^{13}C-NMR studies allowed some assessment of compartmental equilibration of glycine and of the relative rates of glycine to serine versus serine to glycine reactions. The serine isotopomers are derived from the serine formed from glycine during the incubation. Therefore, the fractions of ^{13}C-labelled intracellular glycine and m-THF utilised by GC and SHMT are predicted from these isotopomers. GC-MS measures the average fraction of ^{13}C-labelled glycine in a whole sample, which includes both intracellular and extracellular glycine. Identical values of ^{13}C-labelled glycine were determined by ^{13}C-NMR and GC-MS. This indicates that equilibration of intracellular and extracellular glycine must have occurred, otherwise the unlabelled glycine formed intracellularly would have reduced the intracellular fraction of ^{13}C-labelled glycine available for serine formation. Therefore, F_{gly} calculated from the serine isotopomers would have been lower than the fraction measured by GC-MS. Comparison of intracellular and extracellular glycine confirmed rapid transport of glycine into the proximal tubules giving a 4-fold higher concentration of intracellular glycine (data not shown) similar to previous investigations by Weinberg et al. [2].

In these freshly purified proximal tubules, serine formation from glycine appears to be the preferred direction of SHMT and GC. Specific fractions for total ^{13}C-labelled glycine and serine are maintained during incubation. When glycine was 93%, 68% or 42% ^{13}C-labelled (Table 2), serine was 46.4%, 46.7% and 41% ^{13}C-labelled respectively (Table 3). If the forward and reverse reactions of GC and SHMT were rapid and equivalent, the fraction of labelled glycine and the total fraction of labelled serine would become equivalent. Clearly, the total fraction ^{13}C-label did not equilibrate between glycine and serine. In contrast to our renal tubule experiments, studies of purified liver SHMT indicate glycine formation from serine is the preferred direction of SHMT [15,32]. Two conditions used in this study should drive the reaction from glycine to serine. Firstly, the reaction has not reached equilibrium at 20 min. Therefore a concentration of 5 mM glycine, which approximates the SHMT K_m for glycine in liver, should drive the reaction to serine, since the concentration of serine formed remains well below its K_m. Secondly, in the mitochondria the association of GC with SHMT should immediately scavenge THF formed by SHMT and convert it back to m-THF, continually driving the reaction towards serine. Consistent with this, in vivo studies found ^{14}C label transferred from glycine to serine at twice the rate of serine to glycine [33]. Conversion of serine to glycine was not detected in isolated rabbit proximal tubules [2]. Other indirect evidence includes a more than two-fold faster rate of ammonia released when cortical slices were incubated with 10 mM glycine (21.0 μmol · h^{-1} per g kidney) compared with 10 mM serine or no substrate (8.8 μmol · h^{-1} per g kidney) [16]. Related evidence is that serine failed to protect proximal tubules against hypoxic injury [34], compared with glycine or glutathione [35]. If glycine was readily formed from serine, newly formed glycine should provide protection against hypoxic injury and the rate of ammonia formation should be similar when glycine or serine are single substrates.

We also observed rapid formation of unlabelled glycine and serine at the start of the incubation. F_{gly} was consistently lower than the initial fraction of ^{13}C-labelled glycine without significant change during further incubation (Tables 1 and 2). Serine was less than 50% labelled, even with 100% [2-^{13}C]glycine as sole substrate (Table 3). Multiple washes without glycine or serine during tubule preparation should have minimised the unlabelled glycine or serine present at the start of incubation. This has been demonstrated in rabbit proximal tubules where intracellular amino acids were washed out during preparation [2]. In addition, the very small intracellular volume of the tubules, compared to the volume of incubation medium, would require a very high intracellular unlabelled glycine concentration to alter the fraction of added labelled glycine from an initial concentration of 5 mM. Weinberg et al. [2] incubated purified rabbit proximal tubules with 2 mM [2-^{13}C]glycine and unlabelled 5 mM glucose, 4.5 mM sodium lactate, 1 mM alanine, and 10 mM sodium butyrate for 15, 30 and 60 min, and measured the ^{13}C-enrichment of serine as 30.6 ± 2.0, 42.4 ± 2.1 and 47.3 ± 3.8% respectively. The similarity of their results and ours obtained with glycine as the sole substrate (Table 3) suggests a source of unlabelled serine exists independent of the unlabelled substrates added. Studies on the contribution of proteolysis to glycine and serine formation have given conflicting results.

Low rates of proteolysis, as indicated by amino acid release, have been reported in rabbit proximal tubules [2]. In contrast, serine formation from tubular protein in isolated rat cortical tubules, was estimated as 34.3 μmol · g dry wt^{-1} per h, a value which could readily account for serine formation measured without added substrates (30 μmol · g dry wt^{-1} per h) [29]. In the latter study, the rates of serine and glycine formation (30 and 8.2 μmol · g dry wt^{-1} per h respectively) reported without added substrates [29], are sufficient to account for the decreased fraction of ^{13}C-labelled serine and glycine measured in this paper. The potential for de novo glycine formation by the kidney has been demonstrated in normal rats by Lowry et al. [36], who observed that a net uptake of glycine of 208 ± 83 nmol · min^{-1} per animal became a net release of 463 ± 184 nmol · min^{-1} per animal when animals were given cysteamine to inhibit GC, suggesting that free glycine uptake accounted for less than 50% of available intracellular glycine.

The ability to follow glycine incorporation into m-THF, using F_m and $F_{m(GC)}$, is unique to serine isotopomer analysis. This will allow potential precursors of m-THF to be added with glycine to assess their ability to compete with glycine as a source of the methyl group in m-THF. In this study, an initial F_{gly} of 100%, 75% and 50% gave $F_{m(GC)}$ of 99.0, 96.8 and 96.4% respectively (Table 2), suggesting that glycine was the major carbon donor for THF. This agrees with previous investigations of the stoichiometry of serine formation in rat kidney cortical slices [16]. However, as the incubation medium contained only free glycine and no other substrates, our study may have exaggerated the contribution of glycine as a methyl donor to THF.

In conclusion, ^{13}C-NMR detected rapid conversion of glycine to serine without significant formation of other products. Three unique serine isotopomers were detected by ^{13}C-NMR, which confirmed the expected mitochondrial coupling of SHMT and GC in fresh rat renal proximal tubules and allowed the unlabelled fractions of glycine and m-THF to be predicted. The formation of serine from glycine by SHMT and GC was much faster than the reverse reaction. Serine isotopomer analysis and GC-MS provide an effective method for analysing the metabolic relationships between glycine and serine in situ and for detecting unlabelled glycine, serine and m-THF formation under normal conditions and in disease states.

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